FEMS Microbiology Letters 79 (1991) 69–74

Published by Elsevier
ADONIS 037810979100168V

FEMSLE 04367

Comparison of culture methods for production of *Colletotrichum truncatum* spores for use as a mycoherbicide

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Received 18 September 1990
Accepted 20 November 1990

Key words: Membrane culture; Solid-state culture; Submerged culture; Plackett-Burman; Biocontrol

1. SUMMARY

Four culture systems were compared: (1) dialysis membranes (MF), (2) liquid shake flasks (LF), (3) solid particles with humidity control (SFC), and (4) solid particles without humidity control (SFE). A Plackett-Burman fractional factorial experimental design was employed with 10 total variables. Eight media components, plus two levels of inoculum, were tested in all systems. Other variables were light vs. dark for MF, agitation level for LF, vermiculite vs. rice hulls for SFC, and particle size for SFE. High yeast extract (1 g/l) produced more ($P < 0.01$) spores than low (0.3 g/l) for *all* culture systems. Carbohydrate (sucrose) at 20 g/l produced more spores than at 40 g/l for SFC. $MgSO_4 \cdot 7H_2O$ at 2 g/l was better than none for LF and SFC. For MF, LF, SFC, and SFE, respectively, the overall average numbers of spores per ml of medium were 0.53, 0.72, 0.28, and 0.073×10^7, while the highest numbers of spores were 1.25, 2.75, 1.52, and 0.46×10^7. Thus, the method of production cannot be decided at this stage but must await further studies of recovery and storage.

2. INTRODUCTION

Colletotrichum species are the causes of diseases of many different plants of commercial and horticultural interest, some of which are weeds. *C. gloeosporoides* f sp. *aeschynomene*, sold under the brand name Collego ® is an already commercialized species for use as a mycoherbicide against northern jointvetch (*Aeschynomene virginica*) [1,2]. The development of Collego ® has been described [3,4]. Among the many *Colletotrichum* species studied, the effects of many variables (both chemical and environmental) upon growth, sporulation, and germination have been investigated. Most of the studies have employed agar-plate cultures.

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C. truncatum, a pathogen of hemp sesbania (*Sesbania exaltata*) [5], which is a weed of rice and cotton is reported in this investigation. Inundative [6] rather than classical [7] control of this weed is the ultimate goal. Consequently, production of large quantities of conidia of this spore-forming pathogen is required for application to the weeds in the field as they emerge in the spring. C.D. Boyette, of the Southern Weed Science Laboratory, USDA, Stoneville, Mississippi, discovered this organism and evaluated its bioherbicidal potential [8]. The fungus sporulated poorly in liquid culture compared to culture on agar. Preliminary tests were run that established spore production could be achieved on agar, on membranes over a pad soaked with liquid media, in liquid shake flasks, and on solid particles soaked with liquid media. These lab-scale methods were chosen to represent elemental features characteristic of possible full-size production systems. Selection of media ingredients was based on comparison of growth and spore production on 22 different agar-slant media. Other parameters varied in this comparison were inoculum level, solid particle kind (vermiculite or rice hulls), solid particle size, liquid agitation level, and culture in the light or dark.

3. MATERIALS AND METHODS

3.1. Cultures

A culture of *C. truncatum* was obtained from C.D. Boyette and deposited in the NRRC Culture Collection as NRRL 13737. It has been transferred monthly on potato dextrose agar slants [9]. During each month, weekly transfers were made on media containing 0.1 g/l Tastone 154 yeast extract (Universal Foods Corp.); 0.5 g/l Pharmamedia (Traders Protein); 20 g/l glucose, and 15 g/l agar (Difco). Inoculum for three different culture methods was produced similarly, using this agar media. Five ml of 0.01% Triton was added to a slant, mixed with a Vortex mixer to suspend the spores, then either 0.1 ml, 0.5 ml, or 2.5 ml was added to another slant, a 90-mm petri plate with 30 ml agar media or a 2800-ml Fernbach flask with 250 ml agar media. Incubation was for 4 days, after which either 5, 14, or 70 ml of 0.01% Triton, respectively, was added to harvest the desired volume of inoculum. A sterilized, stiff artist's brush was used to brush the agar surface of the plate and Fernbach flask to release the maximum number of spores. Inoculum for these experiments was $3.7 \pm 1.3 \times 10^7$ per ml based on microscopic counts and $2.1 \pm 0.9 \times 10^7$ per ml based on plate counts. Relatively large amounts of inoculum were used to achieve microcycle conidiation, a process in which conidiation occurs after minimal mycelial growth and acervuli are crowded as much as possible [10].

3.2. Experimental design

A Plackett-Burman fractional factorial experimental design [11] was employed with two levels each of 10 variables in an experimental set of 12. Ten variables each at 2 levels would require 1024 (2^{10}) trials for a complete experiment. The fractional factorial design allows estimation of the simple effects of the 10 main variables in a fraction of the 1024 possible trials. The ability to examine interactions among the main effects is lost, however. The Plackett-Burman design allows efficient and unbiased estimation of the 10 min effects in 12 trials. Table 3 lists the variables under 'Factor' and specific levels can be determined under the conclusion column. Two sets of 12 trials were made in duplicate. In the first set, the arrangement in Table 1 was used. In the second set, the opposite arrangement was used; i.e., where there is a (+) in Table 1, the (−) value of the variable was used, and where there is a (−) in Table 1, the (+) value of the variable was used.

3.3. Inoculum variables and culture method

The inoculum variable was at two levels: 1.7% or 5.1% of 100 ml liquid media in 250-ml Erlenmeyer flask for liquid culture (LF). Incubation was at 25 °C either on a 5.08 cm (2-inch) eccentricity shaker at 300 rpm or a 3.81 cm (1.5-inch) eccentricity shaker at 200 rpm under fluorescent lights.

For membrane cultures (MF), 20 ml of well-mixed sterile medium was added to a sterilized filter pad (F.R. Hormann & Co.) cut to fit a 90-mm plastic petri dish. Then a wet, sterilized,

precut (circular) cellophane dialysis membrane was placed on top of the pad, followed by either 0.1 ml or 0.5 ml of the inoculum described above. A bent, heated and cooled platinum wire was used to spread the inoculum over the entire cellophane surface. Incubation was at 25 °C, either in the dark or under constant fluorescent light.

For both kinds of solid-state culture, inoculum was added to the sterile medium at 1.7% or 5.1% of the volume needed to obtain 100% saturation. Vermiculite required ca. 26.6 ml of inoculated medium per 10 g while rice hulls required ca. 28.7 ml of inoculated medium per 10 g. Ten g of solids was used in both 500-ml Erlenmeyer flasks (SFE) or in 50 × 90 mm diameter crystal dishes (SFC) with a filter pad (50 mm diameter) saturated with sterile distilled water glued to the center of a 100-mm-diameter petri dish cover. In the SFE experiments, either > 20-mesh or 60–100-mesh vermiculite was used. In the SFC experiment, either vermiculite or rice hulls as received were used. Incubation was at 25 °C under fluorescent lights. All culture systems were incubated for 4 days.

3.4. Analysis

The number of spores was determined microscopically, using a Petroff-Hauser counter.

For membrane fermentations, a cork borer was used to cut a 20-mm-diameter piece from the cellophane membrane. Three ml of 0.01% Triton

Table 2

Replicate spore counts $\times 10^7$/ml after 4 days incubation in MF, LF, and SFC culture systems

Trial no.		MF	LF	SFC
(+) [a]	1	0.23, 0.60	1.61, 1.14	0.15, 0.105
	2	0.50, 0.04	1.48, –	0.025, 0.005
	3	0.12, 0.21	0.59, 0.78	0.07, 0.11
	4	0.44, 0.21	0.61, 0.23	0.055, 0.04
	5	0.065, 0.185	0.19, 0.09	0.04, 0.01
	6	0.19, 0.12	0.025, 0.035	0.025, 0.015
	7	0.33, 0.25	1.88, 1.51	0.05, 0.35
	8	0.03, 0.08	2.16, 3.33	0.39, 0.11
	9	0.025, 0.035	0.43, 0.32	0.135, 0.11
	10	0.05, 0.06	0.47, 0.45	0.38, 0.03
	11	0.05, 0.06	0.14, 0.25	0.08, 0.06
	12	0.075, 0.05	0.18, 0.29	0.125, 0.105
(–) [a]	1	0.47, 0.25	0.46, 0.34	0.05, 0.02
	2	0.37, 0.36	1.10, 0.19	0.02, 0.05
	3	0.005, –	0.05, 0.55	0.05, 0.005
	4	0.09, 0.055	0.05, 0.02	0.02, 0.003
	5	0.31, 0.29	0.27, 0.07	0.135, 0.22
	6	0.01, 0.06	0.28, 0.19	0.135, 0.13
	7	0.11, 0.17	0.34, 0.53	0.013, 0.01
	8	0.04, 0.05	0.02, 0.16	0, 0.015
	9	0.18, 0.46	0.83, 0.37	0.02, 0.02
	10	0.22, 0.22	2.23, 1.09	0.128, 0.135
	11	0.06, 0.18	1.38, 2.06	0.09, 0.05
	12	0.25, 0.27	1.31, 2.49	0.01, 0.015

MF = spore count $\times 10^7$/ml suspension.
LF = spore count $\times 10^7$/ml broth.
SFC = spore count $\times 10^7$/ml suspension.
(+) [a] refers to one Plackett-Burman experimental design, while (–) [a] refers to the opposite design (see text).

Table 1

Plackett-Burman fractional factorial experimental design plus (+) configuration

Factor	Trial no.											
	1	2	3	4	5	6	7	8	9	10	11	12
Glucose vs. sucrose	+	+	–	+	+	+	–	–	–	+	–	–
Sugar concentration	–	+	+	–	+	+	+	–	–	–	+	–
Tastone 154 level	+	–	+	+	–	+	+	+	–	–	–	–
Pharmamedia level	–	+	–	+	+	–	+	+	+	–	–	–
KH_2PO_4	–	–	+	–	+	+	–	+	+	+	–	–
$MgSO_4 \cdot 7H_2O$	–	–	–	+	–	+	+	–	+	+	+	–
$CaCO_3$	+	–	–	–	+	–	+	+	–	+	+	–
Tap water vs distilled	+	+	–	–	–	+	–	+	+	–	+	–
Variable [a]	+	+	+	–	–	–	+	–	+	+	–	–
Inoculum level	–	+	+	+	–	–	–	+	–	+	–	–
Dummy variable	+	–	+	+	+	–	–	–	+	–	+	–

[a] Light vs. dark for MF; 2′, 300 rpm vs. 1.5′, 200 rpm for LF; and vermiculite vs. rice hulls for SFC.

was added and the membrane was brushed to dislodge spores, making a suspension which was counted. The SFE or SFC samples were weighed, corrected for any evaporative losses, and diluted the same as that employed for the MF spore count with 0.01% Triton. The common dilution was 3 ml 0.01% Triton per ml of original liquid medium.

4. RESULTS AND DISCUSSION

This study was performed in order to compare membrane, liquid, and solid-state culture. It was done in the manner chosen to provide both a wide array of 24 comparative conditions as well as a systematic look at the media factors studied. The

Table 3

Standardized effect [a] of each factor in each culture system on spore count

Factor	Culture system	Standardized effect [a] of factor	sig. [b]	Conclusion [c]
Glucose vs. sucrose	MF	0.09	0.57	Glu = Suc
	LF	−0.04	0.85	Glu = Suc
	SFC	−0.64	< 0.01	Glu < Suc
Sugar concentration	MF	0.10	0.50	40 g/l = 20 g/l
	LF	−0.04	0.82	40 g/l = 20 g/l
	SFC	−0.58	< 0.01	40 g/l < 20 g/l
Tastone 154 level	MF	0.49	< 0.01	0.3 g/l < 1 g/l
	LF	0.72	< 0.01	0.3 g/l < 1 g/l
	SFC	0.33	0.03	0.3 g/l < 1 g/l
Pharmamedia level	MF	0.01	0.99	1.5 g/l = 0.5 g/l
	LF	0.56	< 0.01	0.5 g/l < 1.5 g/l
	SFC	0.18	0.22	1.5 g/l = 0.5 g/l
KH_2PO_4	MF	−0.15	0.34	1 g/l = 0, no effect
	LF	0.11	0.50	1 g/l = 0, no effect
	SFC	−0.19	0.19	1 g/l = 0, no effect
$MgSO_4 \cdot 7H_2O$	MF	0.09	0.55	2 g/l = 0, no effect
	LF	0.32	0.04	2 g/l > 0
	SFC	0.29	0.06	2 g/l > 0
$CaCO_3$	MF	−0.02	0.87	5 g/l = 0, no effect
	LF	0.23	0.16	5 g/l = 0, no effect
	SFC	−0.02	0.87	5 g/l = 0, no effect
Tap water vs. distilled	MF	−0.05	0.69	tap = distilled
	LF	0.22	0.14	tap = distilled
	SFC	0.29	0.05	tap > distilled
Light vs. dark	MF	−0.06	0.69	No effect
2′, 300 rpm vs. 1.5′, 200 rpm	LF	0.15	0.30	No effect
vermiculite vs. rice hulls	SFC	0.02	0.87	No effect
Inoculum level	MF	0.11	0.47	0.1 ml/plate = 0.5 ml/plate
	LF	0.07	0.58	5.1% = 1.7%
	SFC	0.11	0.45	5.1% = 1.7% (of the liquid)

[a] Standardized effect = (actual count − mean)/overall standard deviation.
MF: mean = 0.180, S.D. = 0.145; LF: mean = 0.7333, S.D. = 0.580; SFC: mean = 0.069, S.D. = 0.050.
[b] sig. = probability that the effect may be zero.
[c] Based on spore production.

Plackett-Burman design allows direct estimation of main effects, but evaluation of interactions is complicated. In this analysis, an overall analysis of variance was run with a model containing each factor. Preliminary analysis found no significant effects of replication or block by replication interaction. We concluded that any main effect which was significant in this overall analysis was a real effect. Main effects which appeared as significant in one scenario (Table 1 configuration) but not the other (opposite of Table 1) were indicative of possible underlying interactions. In this work, very similar Plackett-Burman designs were used on four different reactor systems so there are other evaluations possible, namely: which variables are important for each reactor system? Are any variables important in all reactor systems?

The spore count data are presented in Table 2. These data were analyzed to give mean values for each of the factors/variables for each of the reactor systems. In order to compare reactor systems, the average value for each main effect was standardized by subtracting the overall mean for each block (plus (+) or minus (−)) and then dividing by the standard deviation for each reactor system. These standardized effects are listed in Table 3. A low value in the sig. column of this table indicates that the standardized effect is significant. The sign (+ or −) of the standardized effect of the factor tells the direction of the effect. The higher the numeric value, the larger the effect. Thus, in the case of the SFC reactor: sucrose at 20 g/l, Tastone 154 at 1 g/l, $MgSO_4 \cdot 7H_2O$ at 2 g/l, and tap rather than distilled water are the recommended factor levels. Also, Tastone 154 at high level was significant in all the reactors.

Why are some variables more important in one reactor type but not in another? One explanation is that Pharmamedia has components which are not soluble and, therefore, in an agitated LF, the insolubles can be readily acted upon by extracellular enzymes without the more extreme diffusion distances which would be encountered in SF reactors or complete hindrance by dialysis membrane in MF. A similar insoluble medium component is $CaCO_3$. Another possible explanation is the inability of the larger molecular mass enzymes or substrates to diffuse through the membrane or spread over the solid surfaces. Further, the very recent work of Cascino et al. [12] with *C. gloeosporioides* illustrates the effects of substrate limitations on spore production and may explain the severe differences from one reactor type to another for the same medium trial.

Another comparison is of the average spore production based upon a uniform quantity of media used in each reactor type. For 24 reactors each of MF, LF, SFE, and SFC, the average number of spores was 0.53, 0.72, 0.073, and 0.28 $\times 10^7$ spores/ml, respectively. MF, LF, and SFC gave results of the same order of magnitude, while the SFE spore production was an order of magnitude lower probably due to drying out during the 4th day of incubation. A similar result is apparent from the spore counts of the *best* of the 24 reactors for MF, LF, SFE, and SFC, which were 1.25, 2.75, 0.46, and 1.52 $\times 10^7$ spores/ml, respectively.

5. CONCLUSIONS

Since the overall average and best spore count per ml media for MF, LF, and SFC cultures are each of the same order of magnitude, a choice of culture methods cannot be made at this time. This similarity of results of spore production per ml media for different culture methods was observed also by Slade et al. [13] in a comparison of liquid shake flask culture and surface culture on agar in microplate wells. It should be noted that this similarity may not apply to other products such as enzymes or primary and secondary metabolites. It should *not* be concluded that because there is a similar order of magnitude between MF, LF, and SFC that there is little hope for significant improvement because there *was* an order of magnitude improvement between SFE and SFC which was achieved simply by increasing percent RH with the water-soaked pad.

The fact that we cannot choose an optimum reactor system at this time is not necessarily a real dilemma except that it means more work. The fact that all three options are still open means that a range of production technologies remain available and consequently a similar variety of spore re-

covery and storage techniques require investigation.

ACKNOWLEDGEMENTS

The technical assistance of Thomas Larsen and the helpful discussions of Mark Jackson are greatly appreciated.

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